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MiR-222-3p promotes the proliferation, migration and invasion of papillary thyroid carcinoma cells through targeting SLC4A4

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Summary. Objective. An increasing number of studies indicate that miR-222-3p is upregulated in various cancers and can regulate tumor progression. This study aimed to explore the regulatory mechanism of miR-222-3p in papillary thyroid carcinoma (PTC).

Methods. TCGA database was used to dig differentially expressed miRNAs and mRNAs in PTC tissue. Relevant references were searched to determine target miRNA. StarBase, TargetScan and miRDB were applied to predict mRNAs that had binding sites with the target miRNA. Then, the mRNAs were intersected with differentially downregulated mRNAs in TCGA to determine the target mRNA. qRT-PCR was exerted to evaluate gene expression of miR-222-3p and SLC4A4 in PTC. Western blot was performed out to evaluate the protein expression of SLC4A4 in PTC cells. CCK-8, wound healing assay and cell invasion assay were undertaken to observe the proliferative, migratory, and invasive abilities of PTC cells. Dual-luciferase assay was employed to test the binding relationship between miR-222-3p and SLC4A4.

Results. MiR-222-3p was highly expressed in PTC while SLC4A4 was lowly expressed. Moreover, miR-222-3p was able to promote the proliferation, invasion, and migration of PTC cells. SLC4A4 was able to reverse these promotive effects of miR-222-3p.

Conclusion. MiR-222-3p can promote the proliferation, migration and invasion of PTC cells through targeting SLC4A4. MiR-222-3p is expected to be a molecular therapeutic target for PTC patients.

Key words: miR-222-3p, SLC4A4, PTC, Proliferation, Migration, Invasion

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Introduction

Thyroid carcinoma (THCA) is one of the most common malignant tumors in the endocrine system (Liebner and Shah, 2011), including 4 histological types: follicular thyroid carcinoma, papillary thyroid carcinoma (PTC), anaplastic thyroid carcinoma and Hürthle carcinoma (Chen et al., 2009). PTC is the major type, accounting for 75%-85% of all THCA cases (Geraldo and Kimura, 2015). PTC is usually curative and has a 5-year survival of more than 95% (Hay et al., 2002). However, it sometimes differentiates to more aggressive and fetal THCA, and about 30% of patients have been observed to relapse (Yoruker et al., 2016). Therefore, it is a necessity to analyze the molecular characteristics of this cancer for potential therapeutic targets and effective treatment.

MicroRNAs (miRNAs) are highly conserved endogenous non-coding RNAs with a length of 22 nucleotides. They can bind to the 3'-untranslated regions (3'-UTR) of mRNAs as a negative regulator to mediate translational suppression or degradation of the target mRNA (Yekta et al., 2004; Iorio et al., 2005). In recent years, with intensive studies performed on miRNAs, it has been demonstrated that miRNAs can play a key regulatory role in the progression of THCA. For example, Zhenglin Wang found that miR-873-5p suppresses the migration and invasion of THCA cells through regulating the expression of CXCL16 (Wang et al., 2020). Y P Shi also represented that miR-17-5p can promote the proliferation and autophagy of THCA cells while inhibiting cell apoptosis through targeting PTEN (Shi et al., 2020). MiRNA miR-222-3p researched in this study has been proved to be capable of regulating the progression of various cancers, including diffuse large B-cell lymphoma (Sun et al., 2019) and epithelial ovarian cancer (Fu et al., 2016). So far, miR-222-3p is found to be an underlying biomarker for PTC lymphatic metastasis (Jiang et al., 2020). Enhanced miR-222-3p

expression stimulates TPC cell proliferation, while knock-down of miR-222-3p can inhibit the ability (Zhang et al., 2018). It is elusive whether miR-222-3p stimulates PTC cell migration and invasion via other molecules.

In this study, we testified that miR-222-3p was differentially expressed in PTC. Meanwhile, CCK-8, wound healing assay and cell invasion assay were performed to observe the effect of miR-222-3p on the proliferation, migration and invasion of cancer cells. Finally, the molecular regulatory mechanism of miR-222-3p was deeply explored to provide a theoretical basis for miR-222-3p as a potential therapeutic target for PTC patients.

Materials and methods

Bioinformatics analysis

Expression data of mature miRNAs (Normal: 59, Tumor: 506) and mRNAs (Normal: 58, Tumor: 502) along with clinical data (2019.12.19) of PTC were downloaded from TCGA portal (https://portal.gdc.cancer.gov/). Then, the "edgeR" package was applied to analyze the differentially expressed miRNAs (DEmiRNAs) and mRNAs (DEmRNAs) in normal and tumor groups (|logFC|>2, padj<0.05). References were cited to determine target miRNA. Next, starBase (http://starbase.sysu.edu.cn/), TargetScan (http://www.targetscan.org/vert_72/) and miRDB (http://mirdb.org/) were used to predict downstream target genes of the miRNA to obtain the DEmRNAs which have binding sites with the miRNA. Lastly, correlation analysis was performed to determine the final downstream target mRNA.

Cell culture

Human normal thyroid cell line HTori-3 (BNCC338687) and PTC cell lines TPC-1 (BNCC338689), KTC-1 (BNCC340144) and IHH4 (BNCC340522) were purchased from BeNa Culture Collection (Beijing, China). HTori-3 cell line was cultured in F12K medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY, USA). TPC-1, KTC-1 and IHH4 cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS. All of the cell lines were cultured in a moist incubator containing 5% CO_2 at 37°C.

Cell transfection

TPC-1 and IHH4 cells were inoculated into 6-well plates with 60%-70% confluency. After cells were attached to the walls of the plates, Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect NC-mimic, miR-222-3p-mimic, oe-NC, and oe-SLC4A4 into TPC-1 cell line. NC-inhibitor, miR-222-3p and miR-222-3p-inhibitor were transfected to IHH4 cells. After 48 h of transfection, cells were collected for following research. NC-mimic, miR-222-3p-mimic, oe-NC and oe-SLC4A4 were all obtained from Guangzhou RiboBio Co., LTD. (Guangzhou, China).

qRT-PCR

Following the manufacturer's instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), total RNA was extracted from PTC cell lines. Then, NanoDrop 1000 spectrophotometer (Invitrogen, Carlsbad, CA, USA) was used to assess the purity and concentration of the total RNA. The total RNA was transcribed into complementary DNA (cDNA) through RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Afterwards, qRT-PCR was performed on Applied Biosystems 7500 detection system with SYBR Green Kit (Qiagen, Inc., Valencia, CA, USA). Internal reference for mRNA and miRNA was GAPDH and U6, respectively. $2^{-\Delta\Delta CT}$ method was applied to quantify relative expression of miR-222-3p and SLC4A4. . Primer sequences used in this qRT-PCR are as follows: miR-222-3p: forward: 5'-ACACTCCAGCTGGGAGC TACATCTGGCTACTG-3', reverse: 5' -CTCAACTG GTGTCGTGGA-3'; U6: forward: 5'-CTCGCTTCGG CAGCACA-3', reverse: 5'-AACGCTTCACGAAT TTGCGT-3'; SLC4A4: forward: 5'-TTCACGGAACT GGATGAGCT-3', reverse: 5' -ACTGTGGGAG AGAAAGAAGCC-3'; GAPDH: forward: 5' -GAGTCAACGGATTTGGTCGT-3', reverse: 5'-GACAAGCTTCCCGTTCTCAG-3'.

Western blot

PTC cells were lysed with radioimmunoprecipitation (RIPA) lysis buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Total proteins obtained were quantified with BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Then, equivalent proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Next, the membrane was blocked with 5% skim milk and Tris-buffered saline with Tween 20 (TBST) atroom temperature for 1 h. Primary antibodies SLC4A4 (Cambridge, UK) or GAPDH (Cambridge, UK) were added to the membrane for overnight incubation at 4°C. On the next day, the membrane was incubated with secondary antibody goat anti-rabbit IgG H&L coupled with horseradish peroxidase (HRP) (Abcam, Cambridge, UK) at room temperature for 1 h. Enhanced Chemiluminescence Detection Kit (Sigma-Aldrich, St. Louis, USA) was used to develop protein bands.

CCK-8

PTC cells were inoculated into a 96-well plate with a

density of 2×10^3 cells/well. Then cells were incubated in an incubator containing 5% CO_2 at 37°C. After 0 h, 24 h, 48 h and 72 h of incubation, the plate was added with 10 ul CCK-8 reagent (Dojindo, Tokyo, Japan) and placed in an incubator containing 5% CO_2 at 37°C for 2 h. Thereafter, a microplate reader (SpectraMax M2, Molecular Devices, CA, USA) was applied to measure the absorbance value at 450 nm.

Wound healing assay

PTC cells were inoculated in a 6-well plate with a density of 8×10^5 cells/well and cultured in an incubator containing 5% CO_2 at 37°C. When cell confluency reached 90%, sterile pipette was utilized to generate a linear scratch on the single cell layer. Cells were then rinsed with PBS 2-3 times and grown in serum-free medium. The scratched areas were photographed with an inverted microscope 0 h and 48 h later. Finally, the migration rate of cells was calculated. Migration rate of cells = (scratched area at 0 h –scratched area at 48 h) /scratched area at 0 h.

Transwell invasion assay

The Transwell chamber (EMD Millipore, Billerica, MA, USA) was coated with Matrigel (1.2 mg/ml) and then inoculated with cells (2×10^5 cells/per well). The transfected cells were seeded in the upper chamber with serum-free medium, while the lower chamber was filled with RPMI-1640 medium supplemented with 10% FBS. The Transwell chamber was placed in an incubator with 5% CO_2 at 37°C for 24 h. Afterwards, swabs were used to wipe non-invading cells. The invading cells in the lower chamber were fixed with absolute ethyl alcohol and stained with 0.1% crystal violet at room temperature for 30 min. Finally, cells were counted via an inverted microscope.

Dual-luciferase assay

Wild type (SLC4A4-WT) and mutant SLC4A4 (SLC4A4-MUT) luciferase reporter vectors were obtained from the RiboBio company. PTC cells with 40% -50% confluency were inoculated into a 24-well plate. After cells were incubated overnight, Lipofectamine 2000 was used to transfect SLC4A4-WT/SLC4A4-MUT and miR-222-3p-mimic/NC-mimic into cells. After 48 h of transfection, the cells were collected. The Renilla luciferase was used as an internal normalization standard to normalize firefly luciferase activity. Luciferase reporter analysis system (Promega, Madison, WI, USA) was applied to determine the activity of firefly and renilla luciferase.

Statistical analysis

All experiments were carried out independently at least 3 times. Data were represented with Means ± Standard Deviation (SD) and analyzed on GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). t-test was used to compare the differences between two groups, and analysis of variance was used to compare differences among multiple groups. $P<0.05$ represents a statistically significant difference.

Results

MiR-222-3p is upregulated in PTC

In this study, we performed differential expression analysis on miRNAs in PTC tissue and normal tissue using the edgeR package. A total of 63 DEmiRNAs were obtained (Fig. 1A). In these 63 miRNAs, the expression of miR-222-3p was significantly upregulated in PTC (Fig. 1B). Meanwhile, several references testified that miR-222-3p is upregulated in many cancers (Liu et al.,

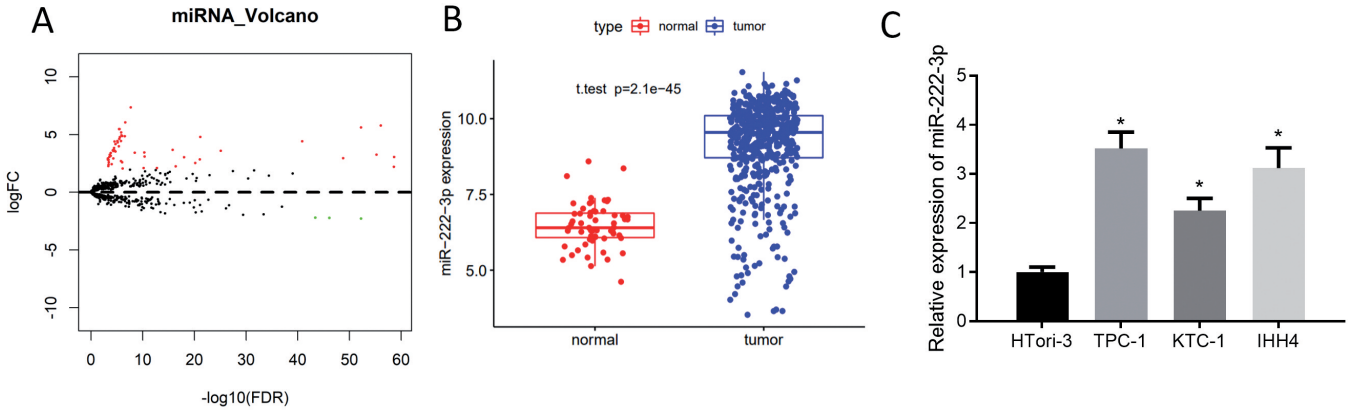

Fig. 1. MiR-222-3p is upregulated in PTC. **A.** Volcano plot of DEmiRNAs in normal and tumor groups in TCGA-PTC dataset, with red dots representing the upregulated miRNAs in tumor group and black dots representing the downregulated miRNAs in tumor group. **B.** The expression of miR-222-3p in PTC samples and normal samples in TCGA, with red boxplot representing normal samples and blueboxplot representing tumor samples. **C.** qRT-PCR was used to detect the expression of miR-222-3p in thyroid cell line HTori-3 and PTC cell lines TPC-1, KTC-1 and IHH4. * represents $p<0.05$.

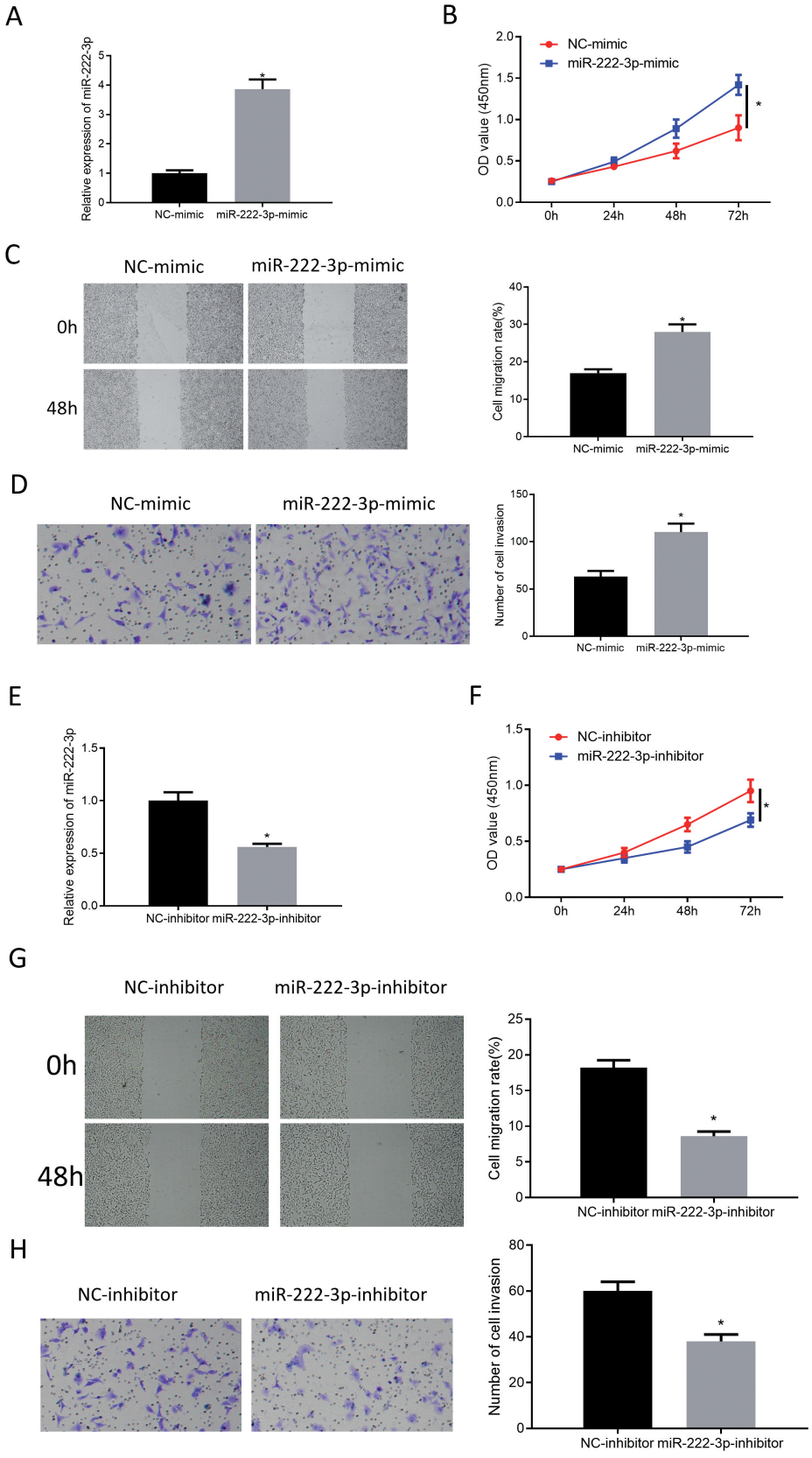

Fig. 2. MiR-222-3p promotes the proliferation, migration and invasion of PTC cells. **A.** qRT-PCR was used to detect the expression of miR-222-3p in TPC-1 cells after miR-222-3p mimic/NC mimic was transfected. **B.** CCK-8 was used to detect the proliferative ability of TPC-1 cells upon miR-222-3p overexpression. **C.** Wound healing assay was used to detect the migratory ability of TPC-1 cells upon miR-222-3p overexpression (×40). **D.** Transwell assay was used to detect the invasive ability of TPC-1 cells upon miR-222-3p overexpression (×100). **E.** After transfection with miR-222-3p inhibitor/NC inhibitor, the expression of miR-222-3p in IHH4 cells was detected by qRT-PCR. **F.** CCK-8 detected the proliferation ability of IHH4 cells after inhibition of miR-222-3p expression. **G.** The migration ability of IHH4 cells after downregulation of miR-222-3p was detected by wound healing assay (×40). **H.** Inhibition of IHH4 cell invasion by Transwell assay after miR-222-3p overexpression (×100). * represents $p<0.05$.

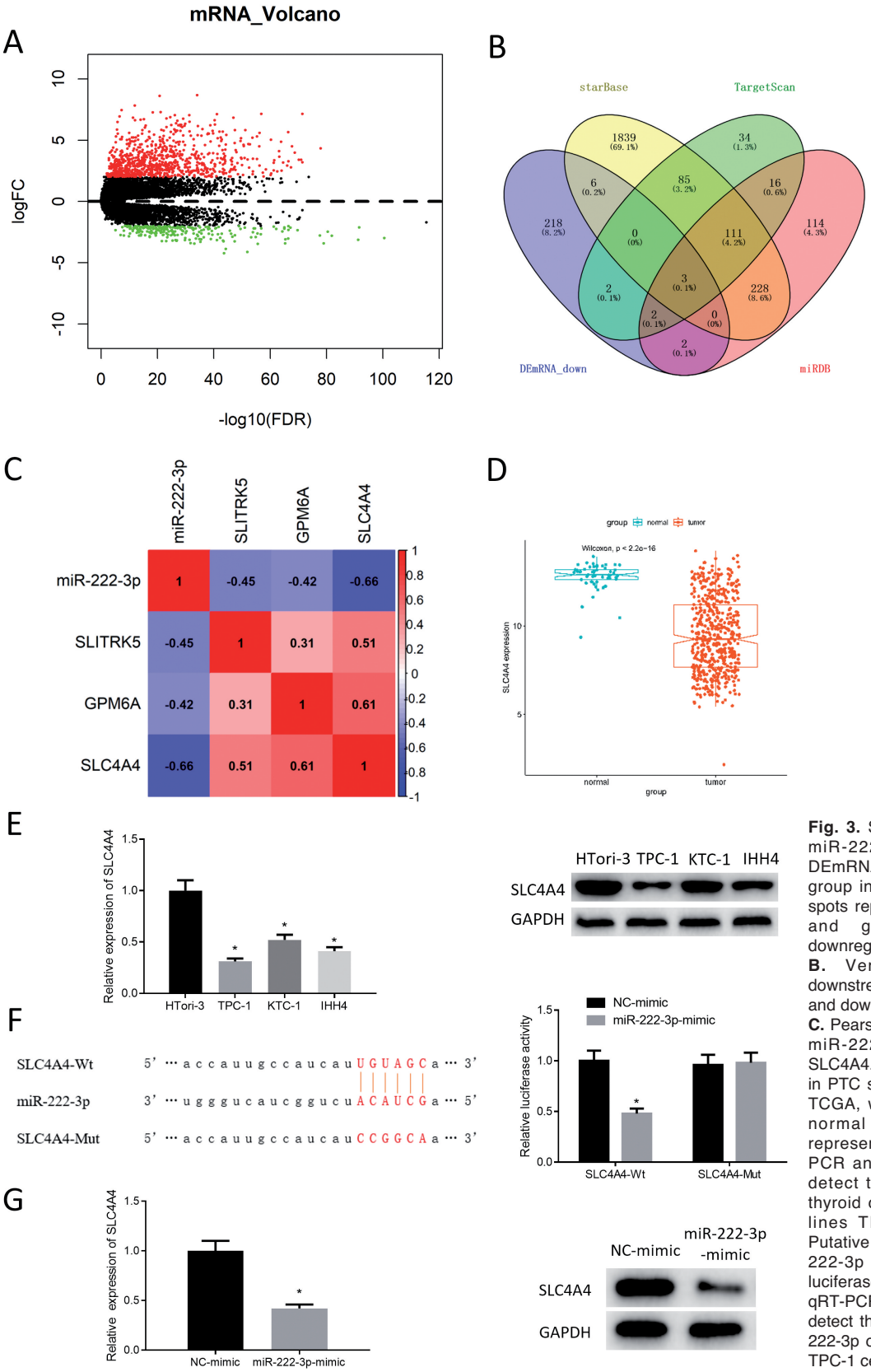

Fig. 3. SLC4A4 is the direct target of miR-222-3p. **A.** Volcano plot of DEmRNAs in normal group and tumor group in TCGA-PTC dataset, with red spots representing upregulated mRNAs and green spots representing downregulated mRNAs in tumor group. **B.** Venn diagram of predicted downstream target genes of miR-222-3p and downregulated DEmRNAs in TCGA. **C.** Pearson correlation analysis between miR-222-3p and SLITRK5/GPM6A/SLC4A4. **D.** The expression of SLC4A4 in PTC samples and normal samples in TCGA, with blue boxplot representing normal samples and red boxplot representing tumor samples. **E.** qRT-PCR and western blot were used to detect the expression of SLC4A4 in thyroid cell line HTori-3 and PTC cell lines TPC-1, KTC-1 and IHH4. **F.** Putative targeted binding sites of miR-222-3p on SLC4A4 3'UTR and dual-luciferase assay used for validation. **G.** qRT-PCR and western blot were used to detect the effect of overexpressed miR-222-3p on the expression of SLC4A4 in TPC-1 cells. * represents p<0.05.

2014; Liu et al., 2019; Tan et al., 2018) and can promote the proliferation, migration and invasion of cancer cells. Therefore, we chose miR-222-3p as the target miRNA for research in this study. To further test the results of bioinformatics analysis, we observed the expression of miR-222-3p in thyroid cell line HTori-3 and PTC cell lines TPC-1, KTC-1 and IHH4. The results showed that compared with HTori-3 cell line, the expression of miR-222-3p was upregulated in TPC-1, KTC-1 and IHH4 cell lines, especially in TPC-1 (Fig. 1C). Hence, we chose the TPC-1 and IHH4 cell lines for following relevant research.

MiR-222-3p promotes the proliferation, migration and invasion of PTC cells

Then, in order to evaluate whether miR-222-3p could regulate the progression of PTC, we transfected miR-222-3p-mimic in TPC-1 cells, and miR-222-3p mimic was transfected to IHH4 cells. MiR-222-3p was significantly up-regulated in TPC-1 cells transfected with miR-222-3p mimic as tested by qRT-PCR (Fig. 2A). MiR-222-3p expression level was decreased in IHH4 cells, significantly (Fig. 2E). We observed the effect of miR-222-3p expression changes on the proliferation, migration and invasion of TPC-1 cells through CCK-8, wound healing and cell invasion assays. Results showed that overexpressed miR-222-3p significantly promoted the proliferation of TPC-1 (Fig. 2B), meanwhile, it strengthened the migratory and invasive ability of TPC-1 cells (Fig. 2C-D). Downregulating miR-222-3p expression hampered IHH4 cell proliferation, migration and invasion (Fig. 2F-H). These data indicated that miR-222-3p was able to

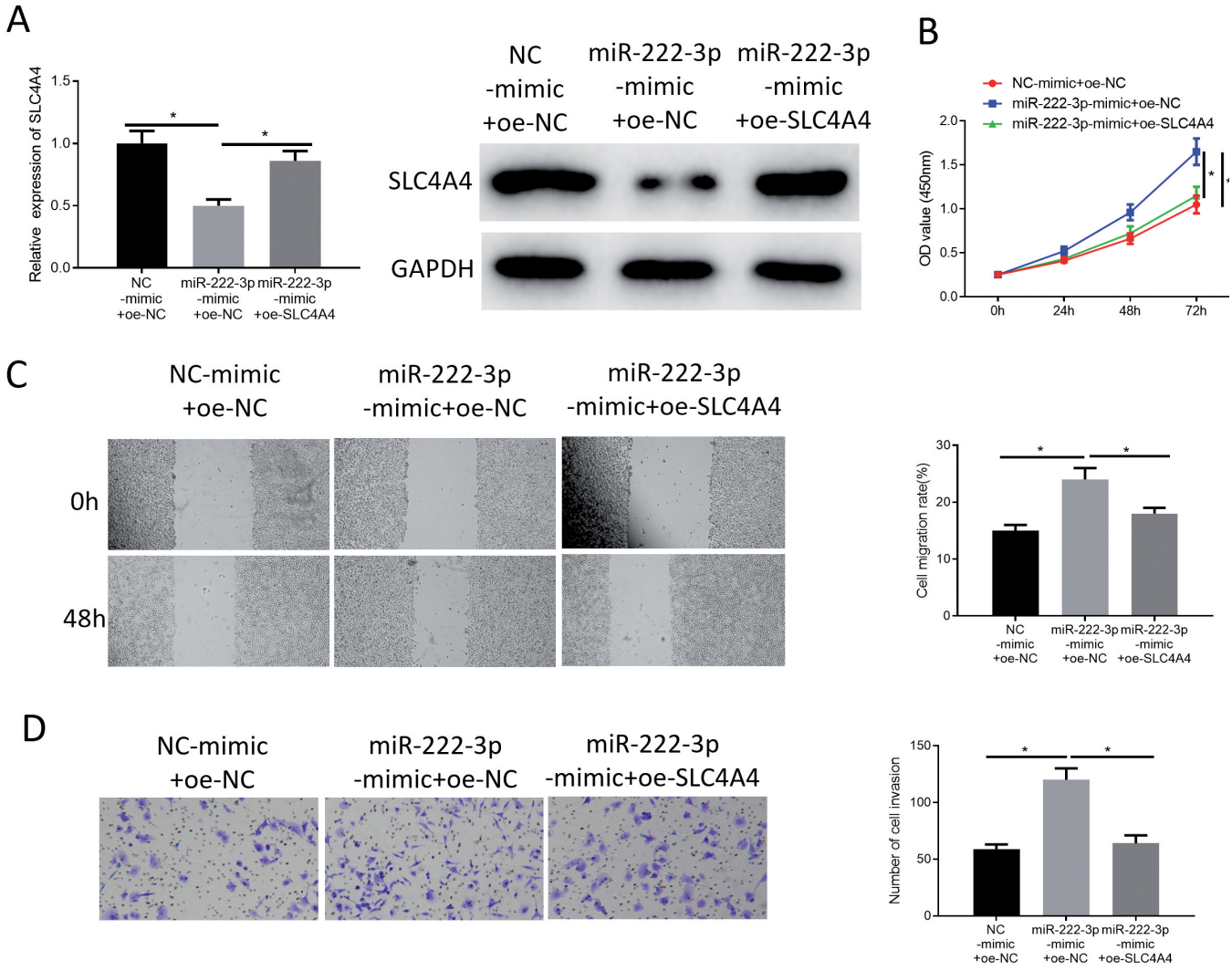

Fig. 4. SLC4A4 reverses the promotive effect of miR-222-3p on the proliferation, migration and invasion of PTC cells. **A.** qRT-PCR and western blot were used to detect the expression of SLC4A4 in TPC-1 cells in 3 groups (NC-mimic+oe-NC, miR-222-3p-mimic+oe-NC, miR-222-3p-mimic+oe-SLC4A4). **B.** CCK-8 assay was used to detect the proliferative ability of TPC-1 cells in 3 groups. **C.** Wound healing assay was used to detect the migratory ability of TPC-1 cells in 3 groups (×40). **D.** Transwell invasion assay was used to detect the invasive ability of TPC-1 cells in 3 groups (×100); * represents $p<0.05$.

promote the proliferation, migration and invasion of PTC cells.

SLC4A4 is one of the target genes of miR-222-3p in PTC

In order to further research the regulatory mechanism of miR-222-3p in PTC, we firstly analyzed DEmRNAs in PTC tissue and normal tissue in the TCGA database by edgeR. Finally, 1,093 DEmRNAs were obtained, in which the expression of 862 mRNAs was upregulated and the expression of 231 mRNAs was downregulated (Fig. 3A). Thereafter, we used starBase, TargetScan, and miRDB to predict downstream target genes of miR-222-3p. Predicted target genes were then intersected with 231 differentially downregulated mRNAs to obtain 3 DEmRNAs which had binding sites with miR-222-3p (SLITRK5, GPM6A, SLC4A4) (Fig. 3B). Through Pearson correlation analysis, we found that SLC4A4 was negatively correlated with miR-222-3p with the highest correlation coefficient (-0.66) (Fig. 3C). Hence, we chose SLC4A4 as the target mRNA. Downregulated expression of SLC4A4 in PTC tissue in the TCGA database is shown in Fig. 3D. Following this, to test whether SLC4A4 is the target gene of miR-222-3p, we firstly observed the expression of SLC4A4 mRNA and protein in thyroid cell line HTori-3 and PTC cell lines TPC-1, KTC-1 and IHH4 through qRT-PCR and western blot, and verified that the expression of SLC4A4 was significantly downregulated in PTC cells (Fig. 3E). Afterwards, dual-luciferase assay showed that miR-222-3p-mimic was able to remarkably reduce the luciferase activity of SLC4A4-WT reporter while it did not affect the luciferase activity of SLC4A4-MUT reporter. This clarified that miR-222-3p was able to bind to SLC4A4 (Fig. 3F). Moreover, we observed that the expression of SLC4A4 was remarkably downregulated in TPC-1 cells in which miR-222-3p was overexpressed (Fig. 3G). The above results demonstrated that miR-222-3p downregulated SLC4A4 expression in TPC cells.

SLC4A4 reverses the promotive effect of miR-222-3p on the proliferation, migration and invasion of PTC cells

To determine whether SLC4A4 could reverse the promotive effect of miR-222-3p on the proliferation, migration and invasion of PTC cells, NC-mimic+oe-NC, miR-222-3p-mimic+oe-NC, miR-222-3p-mimic+oe-SLC4A4 were set. Afterwards, the expression of SLC4A4 was detected through qRT-PCR and western blot. The results showed that transfecting oe-SLC4A4 was able to reverse the suppressive effect of miR-222-3p on SLC4A4 in TPC-1 cells (Fig. 4A). Then, the changes of the proliferative, migratory and invasive ability of TPC-1 cells in 3 groups were observed by CCK-8, wound healing and cell invasion assays. The results showed that SLC4A4 was able to remarkably reverse the promotive effect of miR-222-3p on the proliferation, migration and invasion of PTC cells (Fig. 4B-D). These results fully clarified that the promotion of miR-222-3p on the proliferation, migration and invasion of PTC cells was partially realized by targeting SLC4A4.

Discussion

In the last decade, more and more investigators have studied miRNAs. These studies have shown that miRNAs play important regulatory roles in a variety of physiological and pathological processes, including cell proliferation, differentiation and apoptosis (Bartel, 2004, 2009; Mendell and Olson, 2012). MiR-222 is encoded on the X chromosome (Xp11.3) (Kim et al., 2009; Zhao et al., 2015). Many studies have shown that upregulation of miR-222-3p can promote tumor progression. For example, Xiaoyan Tan confirmed that the expression of miR-222-3p was markedly upregulated in gastric cancer and overexpressed miR-222-3p could significantly promote the proliferation, migration and invasion of gastric cancer cells (Tan, 2018). After determining that the expression of miR-222-3p was upregulated in endometrial cancer, Binya Liu found that miR-222-3p can promote the proliferation and invasion of endometrial cancer cells through cell biological functional experiments (Liu, 2014). Weijun Chen et al. found that miR-222-3p promotes cell proliferation and inhibits apoptosis by targeting BBC3 in non-small cell lung cancer (Chen and Li, 2020). In this study, we confirmed that miR-222-3p was upregulated in PTC cells. We further found that miR-222-3p was able to promote the proliferation, migration and invasion of PTC cells.

To further explore the molecular mechanism of miR-222-3p in PTC, we applied bioinformatics analysis to predict that SLC4A4 is a downstream target of miR-222-3p. We further verified the result through dual-luciferase reporter gene assay and correlated molecular assays. SLC4A4 (Solute Carrier Family 4 Member 4), a member of the SLC4 family with 26 exons, can encode electrogenic sodium bicarbonate cotransporter 1 (NBCe1) (Abuladze et al., 2000; Alper, 2009; Yang et al., 2020). It has been proved that SLC4A4 plays a key role in human physiological processes, like modulating the excitability of neurons (Majumdar and Bevensee, 2010), modulating the contraction, excitability and electrophysiological rhythmicity of cardiac cells (Khandoudi et al., 2001), and maintaining the activity of various enzymes in pancreatic juice (Soyfoo et al., 2009). In recent years, SLC4A4 has also been validated to play an important regulatory role in cancers. For example, MiR-223-3p stimulates cell proliferation, invasion and migration of clear cell renal cell carcinoma by downregulating SLC4A4 (Xiao et al., 2019). Xiutian Zhang et al. (Zhang et al., 2020) revealed that overexpressing miR-223 downregulates SLC4A4 expression to stimulate proliferation, migration, invasion and angiogenesis of pancreatic cancer. To further observe whether SLC4A4 could regulate the progression of PTC, we designed a rescue assay and identified that SLC4A4 was able to reverse the promotive effect of

miR-222-3p on the proliferation, migration and invasion of PTC cells. These results fully revealed that the regulatory effect of miR-222-3p on PTC cells was realized in part by targeting SLC4A4.

All in all, we verified that the expression of miR-222-3p was upregulated in PTC cells and was able to promote the proliferation, migration and invasion of PTC cells. Meanwhile, miR-222-3p inhibited SLC4A4 expression to stimulate PTC cell progression. This study provides a theoretical reference for miR-222-3p to be a potential therapeutic target for PTC patients.

Ethics approval and consent to participate. Studies did not involve human subjects, so the paper is exempt from ethical committee approval.

Consent for publication. Not applicable.

Availability of data and materials. The data used to support the findings of this study are included within the article. The data and materials in the current study are available from the corresponding author on reasonable request.

Competing interest. The authors declare no conflicts of interest.

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Authors' contributions. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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